C5-(1-Propynyl)-2′-deoxy-Pyrimidines Enhance Mismatch Penalties of DNA:RNA Duplex Formation†

Thomas W. Barnes III and Douglas H. Turner*

Department of Chemistry, University of Rochester, Rochester, New York 14627-0216

Received May 21, 2001; Revised Manuscript Received August 15, 2001

ABSTRACT: UV melting experiments show that C5-(1-propynyl)ation of seven pyrimidines to give a fully propynylated oligodeoxynucleotide (PrODN) heptamer increases the thermodynamic stability of six Watson−Crick paired DNA:RNA duplexes by 8.2 kcal/mol, on average, at 37 °C. About 2.5 kcal/mol of this enhancement is due to long-range cooperativity between the propynylated pyrimidines, Y^p's. On average, penalties for dU^p:rG, dC^p:rA, dU^p:rC, and dC^p:rC mismatches are enhanced by 2.9 kcal/mol in PrODN:RNA duplexes over those in unmodified duplexes. This results in penalties as large as 10 kcal/mol for a single mismatch. Removing a single propyne two base pairs away from a mismatch in a PrODN:RNA duplex eliminates the enhancement in specificity. Evidently, enhanced specificity is directly linked to long-range cooperativity between Y^p's. In most cases, the enhanced specificity is larger for internal than for terminal mismatches. PrODN:RNA duplexes are destabilized by full phosphorothioate backbone substitution to give S-PrODN:RNA duplexes. The S-PrODN:RNA duplexes retain enhanced mismatch penalties, however. These results provide insight for utilizing long-range cooperativity and enhanced specificity to improve nucleic acid based probe and drug design.

Molecular recognition of nucleic acids by nucleic acids provides many opportunities for exploiting the deluge of genome sequence data. For example, applications include antisense therapeutics (*1*−*6*) and microarray assays for diagnostics and transcription profiling (*7*−*13*). These applications rely on the strength and specificity of nucleic acid hybridization. The most common natural bases, A, C, G, T, and U, however, did not evolve to optimize specificity. For example, single mismatches can stabilize an RNA:RNA duplex by 3 kcal/mol or destabilize a duplex by 5 kcal/mol, depending on mismatch identity and local nearest-neighbor interactions (*14*−*21*). Likewise, single mismatches can stabilize a DNA:DNA duplex by 2 kcal/mol or destabilize it by 3 kcal/mol (*22*−*29*). Initial sampling of DNA:RNA duplexes indicates a similar sequence dependence of stabilities (*30*).

The specificity of nucleic acid recognition can be enhanced by utilizing circular oligonucleotides (*31*−*33*) or nucleotide modifications (*34*). C5-(1-propynyl) pyrimidines, Y^p's,[1] are one promising class of modified nucleotide (Figure 1) (*35*−*37*). Propynylated oligodeoxynucleotides, PrODNs, enhance binding to RNA in vitro (*37*−*40*), and knock out both high- and low-copy RNA targets with high levels of potency and specificity by inducing RNase H-catalyzed hydrolysis (*41*−*43*). Even a heptamer has great potency (IC_{50} = 0.3 μM) and specificity in knocking out the large T antigen mRNA of SV40 in a plasmid-based microinjection assay (*44*).

Propynylated pyrimidines participate in long-range cooperative interactions that increase DNA:RNA duplex stability by up to 2.6 kcal/mol at 37 °C for a heptamer (*39*). This cooperativity is eliminated by removing a single propynyl or amino group from the major or minor groove, respectively (*39*). This sensitivity to functional groups suggested that base mismatches might greatly reduce hybridization to propynylated oligodeoxynucleotides, and a 100-fold enhanced preference for dU^p:rA pairing versus dU^p:rG pairing has been observed (*40*). Here, we show that dC:rA, dU:rC, and dC:rC mismatches also destabilize PrODN:RNA duplexes more than DNA:RNA duplexes. This enhancement in specificity is dependent on the long-range cooperativity present in fully propynylated helices with only Watson−Crick pairs. Similar enhancement in specificity is also observed with propynylated stereo-random phosphorothioate oligodeoxynucleotides, S-PrODNs. Thus, propynylated oligodeoxynucleotides can greatly increase specificity in applications such as antisense therapeutics and microarray assays.

† This work was supported by NIH Grants GM22939 and DE07202-10. T.W.B. is a Sherman-Clarke Fellow, an Arnold Weissberger Memorial Fellow, and an Oral Cellular and Molecular Biology Training Scholar.

* To whom correspondence should be addressed. Phone: (716) 275-3207. Fax: (716) 473-6889. E-mail: turner@chem.rochester.edu.

[1] Abbreviations: bp, base pair; C^p, C5-(1-propynyl) deoxyribocytidine; C_T, total strand concentration; EDTA, ethylenediaminetetraacetic acid; eu, entropy units (i.e., cal K^{-1} mol^{-1}); IC_{50}, antisense oligomer concentration at which 50% of target's expression is inhibited after microinjection; NAED, normalized absolute elliptical difference; PrODN, C5-(1-propynyl) oligodeoxyribonucleotide (note that in previous papers this has been abbreviated as PODN); rmsd, root-mean-square deviation; RP-HPLC, reversed-phase high-pressure liquid chromatography; s-PrODN, a C5-(1-propynyl) oligodeoxynucleotide containing a single propynyl deletion; TBE, 100 mM Tris, 90 mM boric acid, and 1 mM ethylenediaminetetraacetic acid; S-PrODN, a C5-(1-propynyl) oligodeoxynucleotide containing full stereo-random phosphorothioate substitutions; TLC, thin-layer chromatography; T_m, melting temperature in degrees celcius; T_M, melting temperature in degrees kelvin; U^p, C5-(1-propynyl) deoxyribouridine; Y^p, C5-(1-propynyl)-substituted deoxyribopyrimidine.

10.1021/bi011033+ CCC: $20.00
Published on Web 09/26/2001

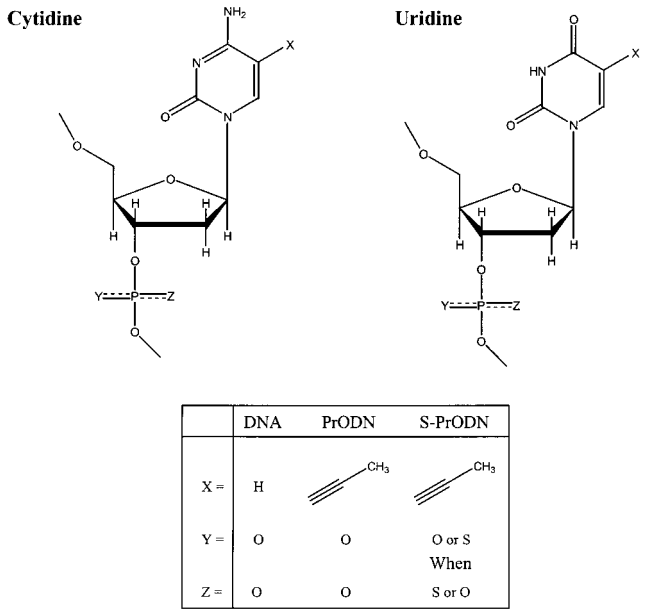

FIGURE 1: Chemical structures of cytosine, uridine, C5-(1-propynyl) cytosine, and C5-(1-propynyl) uracil and backbones discussed in this work. In phosphorothioate PrODNs, S-PrODNs, sulfur substitutes for either a *pro-R* or a *pro-S* nonbridging oxygen within the phosphodiester backbone (i.e., when Y is sulfur, Z is oxygen, and vice versa).

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized by standard chemistry (*45*−*47*) as previously described (*39*), except that the sulfurizing reagent, 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (*48*), purchased from Glen Research, was used for synthesis of C5-(1-propynyl)-ated deoxyribophosphorothioate oligonucleotides (S-PrODNs). Oligonucleotides were purified and analyzed as previously described (*39*). All were >95% pure.

The S-PrODN samples are mixtures of $2^6 = 64$ diastereomers possessing *pro-R* and *pro-S* linkages. When 5′ end labeled with ^{32}P, >95% of each racemic mixture migrates as a uniform band on a 20% denaturing gel in 1 × TBE buffer.

UV Melting. Concentrations of single-stranded RNAs were calculated from high-temperature absorbances at 280 nm and predicted single-strand extinction coefficients (*50*, *51*). Concentrations of single-stranded DNAs and chimeras were calculated from high-temperature absorbances at 260 nm and monomer extinction coefficients (*39*, *52*). The same extinction coefficients were used for phosphorothioate oligonucleotides.

UV melting studies were performed at 260 nm for DNA:RNA duplexes and at 280 nm for PrODN:RNA and S-PrODN:RNA duplexes in 1.0 M NaCl, 0.5 mM Na_2EDTA, and 20 mM sodium cacodylate at pH 7.0, as previously described (*39*). Each melting curve was fit to a non-self-complementary two-state model using Meltwin software (*53*, *54*). Thermodynamic parameters for duplex formation were determined by averaging fits of individual curves and by plotting the reciprocal of the melting temperature, T_M^{-1}, versus $\log(C_T/4)$, where C_T is the total concentration of strands (*55*):

$$T_M^{-1} = (2.303R/\Delta H^\circ)\log(C_T/4) + \Delta S^\circ/\Delta H^\circ \quad (1)$$

Errors were calculated as in Xia et al. (*56*) and references cited therein. When comparing the thermodynamic parameters of duplexes, the results of the T_M^{-1} versus $\log(C_T/4)$ plots were used, and errors were propagated as in Xia et al. (*56*) and references cited therein.

RESULTS

Thermodynamic Parameters of Watson−*Crick Paired Duplexes.* Table 1 lists thermodynamic parameters of duplexes with only Watson−Crick pairs. In most cases, RNA strands are longer than their deoxyribose counterparts to mimic binding sites of nucleic acid therapeutics and probes. Thus, thermodynamic parameters include contributions from stacking of unpaired ribonucleotides. Comparison of duplexes DNA:RNA-1, PrODN:RNA-1^p, and S-PrODN:RNA-1^p, respectively (Table 1), with DNA:RNA-7, PrODN:RNA-7^p, and S-PrODN:RNA-7^p (Table 1) shows that the unpaired ribonucleotides stabilize DNA:RNA-1, PrODN:RNA-1^p, and S-PrODN:RNA-1^p by 2.0, 3.1, and 3.0 kcal/mol, respectively.

Thermodynamic Penalties for Duplexes with Single Mismatches. To determine the thermodynamic penalty for various mismatches in hybrid duplexes, single mismatches were generated by the following substitutions: rA→rC, rG→rC, and rG→rA. The thermodynamic parameters of these duplexes are in Table 2. Thermodynamic parameters for five DNA:RNA and five PrODN:RNA duplexes with single dU:rG pairs have been previously reported (*40*), and they are also listed in Table 2, along with parameters for five S-PrODN:RNA duplexes with similar sequences.

The single mismatches in Table 2 were chosen because they represent three categories, characterized by their impact on the stabilities of DNA:RNA duplexes. First, single dC:rC and dU:rC mismatches destabilize DNA:RNA duplexes in all nearest-neighbor contexts studied thus far (*30*). Second, dC:rA mismatches can stabilize or destabilize DNA:RNA duplexes, depending on nearest-neighbor pairs (*30*). Third, dU:rG pairs stabilize DNA:RNA duplexes in all nearest-neighbor contexts studied thus far (*30*).

Free energy penalties for mismatches, $\Delta\Delta G^\circ_{37}$(MM)'s, were calculated from

$$\Delta\Delta G^\circ_{37}(\text{MM}) = \Delta G^\circ_{37}(\text{duplex-MM}) - \Delta G^\circ_{37}(\text{duplex-WC}) \quad (2)$$

Here, ΔG°_{37}(duplex-MM) is the free energy of the duplex with a single mismatch, and ΔG°_{37}(duplex-WC) is the free energy of the duplex with only Watson−Crick base pairs. Thus, $\Delta\Delta G^\circ_{37}$(MM) depends on both the free energy of the mismatch and the loss in stabilizing free energy of the substituted base pair. The $\Delta\Delta G^\circ_{37}$(MM)'s for replacing dU:rA pairs with dU:rG in DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes range from 0.1 to 0.9, from 2.6 to 4.2, and from 1.3 to 2.4 kcal/mol, respectively (Table 2). The $\Delta\Delta G^\circ_{37}$(MM)'s for replacing dC:rG base pairs with dC:rA mismatches in DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes range from 1.3 to 6.0, from 4.0 to 9.1, and from 2.1 to 7.2 kcal/mol, respectively (Table 2).

Figure 2 summarizes the position and sequence dependence of $\Delta\Delta G^\circ_{37}$(MM)'s for replacing dC:rG and dU:rA pairs with dC:rC and dU:rC mismatches, respectively. The $\Delta\Delta G^\circ_{37}$-(MM)'s for replacing dC:rG base pairs with dC:rC mis-

Table 1: Thermodynamic Parameters of Watson−Crick Paired DNA:RNA, PrODN:RNA, and S-PrODN:RNA Duplexes[a]

			$1/T_m$ plots				curve-fit parameters				predicted
entry	DNA[b,c]	RNA	$-\Delta G^\circ_{37}$ (kcal/mol)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	T_m (°C)	$-\Delta G^\circ_{37}$ (kcal/mol)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	T_m (°C)	$-\Delta G^\circ_{37}$ (kcal/mol)
1	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGAA**AU**5′)**	9.6 ± 0.1	62.8 ± 1.7	171.4 ± 4.9	53.0	9.8 ± 0.2	69.0 ± 2.1	190.7 ± 6.5	52.7	9.5[e] (9.2)[f]
2	d(5′CCUUCUU3′)	**r(3′**GA**GGAAGAA**AU**5′)**	6.8 ± 0.1	57.6 ± 1.5	163.8 ± 4.7	38.2	6.8 ± 0.1	61.4 ± 4.5	176.0 ± 14.3	38.3	7.3[e] (7.2)[f]
3	d(5′CUUCCUU3′)	**r(3′**GA**GAAGGAA**AU**5′)**	6.8 ± 0.1	57.5 ± 2.4	163.6 ± 7.9	38.2	6.8 ± 0.1	62.3 ± 3.0	179.2 ± 9.3	38.1	7.3[e] (7.2)[f]
4	d(5′CUUCCUC3′)	**r(3′**GA**GAAGGAG**AU**5′)**	7.6 ± 0.1	62.0 ± 2.9	175.4 ± 9.5	42.7	7.6 ± 0.2	63.5 ± 2.5	180.3 ± 7.7	42.3	7.9[e] (8.4)[f]
5	d(5′UCUCCUU3′)	**r(3′**GA**AGAGGAA**AU**5′)**	7.0 ± 0.1	54.1 ± 2.1	151.7 ± 6.7	39.8	7.0 ± 0.1	54.2 ± 1.2	152.2 ± 6.4	39.9	7.6[e] (8.1)[f]
6	d(5′CCUCUUU3′)	**r(3′**GA**GGAGAAA**AU**5′)**	6.8 ± 0.1	56.4 ± 2.1	159.7 ± 6.9	38.7	6.9 ± 0.1	54.9 ± 2.3	155.0 ± 7.4	39.0	7.3[e] (7.2)[f]
7	d(5′CCUCCUU3′)	r(3′GGAGGAA5′)	7.6 ± 0.1	53.3 ± 2.0	147.3 ± 6.4	43.3	7.7 ± 0.2	60.0 ± 4.8	168.7 ± 15.5	43.1	
entry	PrODN[b,c]	RNA									
1[p]	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGAA**AU**5′)**	18.4 ± 0.6	89.3 ± 5.4	228.7 ± 15.5	84.3	18.2 ± 1.1	88.3 ± 9.9	226.1 ± 28.6	84.1	18.4[d]
2[p]	d(5′CCUUCUU3′)	**r(3′**GA**GGAAGAA**AU**5′)**	14.9 ± 0.2	84.8 ± 2.1	225.6 ± 6.1	70.8	14.6 ± 0.5	82.8 ± 4.5	219.8 ± 13.1	70.6	15.6[d]
3[p]	d(5′CUUCCUU3′)	**r(3′**GA**GAAGGAA**AU**5′)**	15.4 ± 0.3	79.9 ± 2.5	208.1 ± 7.5	75.6	14.3 ± 0.4	70.3 ± 3.4	180.6 ± 9.6	75.7	15.6[d]
4[p]	d(5′CUUCCUC3′)	**r(3′**GA**GAAGGAG**AU**5′)**	16.2 ± 0.3	86.3 ± 2.7	225.9 ± 7.7	76.1	15.5 ± 0.5	80.4 ± 4.9	209.1 ± 14.3	76.2	16.4[d]
5[p]	d(5′UCUCCUU3′)	**r(3′**GA**AGAGGAA**AU**5′)**	14.7 ± 0.2	77.2 ± 3.0	201.5 ± 8.7	73.7	14.6 ± 0.6	75.9 ± 5.3	197.8 ± 15.1	73.9	15.8[d]
6[p]	d(5′CCUCUUU3′)	**r(3′**GA**GGAGAAA**AU**5′)**	14.2 ± 0.2	78.8 ± 1.6	208.3 ± 4.5	70.7	14.4 ± 0.4	79.5 ± 4.0	210.0 ± 11.6	70.8	15.6[d]
7[p]	d(5′CCUCCUU3′)	r(3′GGAGGAA5′)	15.3 ± 0.4	80.9 ± 4.0	211.6 ± 11.3	74.5	14.6 ± 0.1	74.6 ± 1.9	193.5 ± 5.7	74.7	
entry	S-PrODN	RNA									
S-1[p]	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGAA**AU**5′)**	15.8 ± 0.3	77.8 ± 2.8	200.0 ± 7.8	78.8	14.7 ± 0.8	67.2 ± 7.8	169.3 ± 22.5	79.8	
S-2[p]	d(5′CCUUCUU3′)	**r(3′**GA**GGAAGAA**AU**5′)**	14.1 ± 0.2	76.2 ± 2.3	200.1 ± 6.7	71.3	14.4 ± 0.3	79.9 ± 4.0	207.8 ± 15.0	71.4	
S-3[p]	d(5′CUUCCUU3′)	**r(3′**GA**GAAGGAA**AU**5′)**	14.0 ± 0.2	75.2 ± 1.8	197.1 ± 5.4	71.4	15.4 ± 0.7	88.6 ± 5.5	236.1 ± 15.8	71.5	
S-4[p]	d(5′CUUCCUC3′)	**r(3′**GA**GAAGGAG**AU**5′)**	14.9 ± 0.3	78.9 ± 2.8	206.2 ± 7.9	73.9	14.9 ± 0.5	78.7 ± 5.2	205.6 ± 15.3	73.9	
S-5[p]	d(5′UCUCCUU3′)	**r(3′**GA**AGAGGAA**AU**5′)**	14.5 ± 0.2	74.0 ± 2.3	191.8 ± 6.6	74.5	15.5 ± 0.4	83.1 ± 4.1	218.1 ± 11.9	74.4	
S-6[p]	d(5′CCUCUUU3′)	**r(3′**GA**GGAGAAA**AU**5′)**	14.2 ± 0.4	76.9 ± 3.6	202.3 ± 10.4	71.2	13.5 ± 0.5	70.0 ± 3.9	182.3 ± 11.1	71.2	
S-7[p]	d(5′CCUCCUU3′)	r(3′GGAGGAA5′)	12.8 ± 0.2	61.7 ± 1.9	157.6 ± 5.8	72.2	11.8 ± 0.3	52.9 ± 5.2	208.6 ± 15.3	73.7	

[a] Measured in 1.0 M NaCl, 0.05 mM Na_2EDTA, 20 mM sodium cacodylate, pH 7.0. T_m listed is for 0.1 mM total strand concentration. Errors were calculated as described in ref *56*. [b] Unless otherwise noted, thermodynamic parameters of PrODN:RNA and DNA:RNA duplexes are from ref *40*. [c] Thermodynamic parameters of duplexes DNA:RNA-1 and PrODN:RNA-1[p] are from ref *39*. Thermodynamic parameters of duplexes DNA:RNA-4 and PrODN:RNA-4[p] are previously unreported. [d] Predicted from ref *39* using measured values for unpropynylated duplexes. The rmsd between measured and predicted ΔG°_{37} is 0.74 kcal/mol. [e] Predicted from refs *30* and *68*. The rmsd between measured and predicted ΔG°_{37} is 0.45 kcal/mol. [f] Predicted from refs *68* and *69*. The rmsd between measured and predicted ΔG°_{37} is 0.64 kcal/mol.

matches in DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes range from 3.6 to 6.5, from 4.7 to 10.2, and from 3.6 to 6.6 kcal/mol, respectively (Table 2). The $\Delta\Delta G^\circ_{37}$(MM)'s for replacing dU:rA base pairs with dU:rC mismatches in DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes range from 1.3 to 4.7, from 2.8 to 6.9, and from 1.3 to 5.4 kcal/mol, respectively (Table 2). Propynylation of the DNA strand enhances the penalties for all mismatches when the backbone is phosphodiester, and the average enhancement is 2.9 kcal/mol (Table 2). Phosphorothioate backbone substitutions decrease this average enhancement to 1.1 kcal/mol (Table 2).

Free Energy Penalties Are Larger for dC:rA, dC:rC, and dU:rC Internal Mismatches than for Terminal Mismatches. Table 2 and Figure 2 show that not all $\Delta\Delta G^\circ_{37}$(MM)'s are created equal. The flanking base pairs are one determinant of mismatch penalties, but for dC:rA and dC:rC, the major determinant appears to be position in the duplex. For example, $\Delta\Delta G^\circ_{37}$(dC:rA) at the end of DNA:RNA duplex-1(C:A) is 3.2 kcal/mol, while that within DNA:RNA duplex-3(C:A) is 6.0 kcal/mol. Figure 3 summarizes the average free energy penalties for terminal and internal mismatches. For all oligonucleotide families investigated, dC:rA, dC:rC, and dU:rC mismatches have substantially larger free energy penalties at internal than at terminal positions. In particular, the average $\Delta\Delta G^\circ_{37}$(MM-end) penalties destabilize DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes by 1.3−3.6, 2.8−5.4, and 1.3−3.6 kcal/mol, respectively (Figure 3). The average $\Delta\Delta G^\circ_{37}$(MM-internal) penalties destabilize DNA:RNA, PrODN:RNA, and S-PrODN:RNA duplexes by 3.2−5.4, 5.3−9.4, and 4.0−6.7 kcal/mol, respectively (Figure 3). Full propynylation of DNA strands enhances the penalties of mismatches. Furthermore, the S-PrODN:RNA duplexes retain elevated $\Delta\Delta G^\circ_{37}$(MM)'s, except for terminal dC[p]:rC and dU[p]:rC mismatches (Figure 3).

Effects of Single Propynyl Deletions on Penalties for Mismatches. The thermodynamic parameters of the Watson−Crick paired duplex, d(5′C[p]**C**U[p]C[p]C[p]U[p]U[p]3′)/r(3′GA**GGAGGAA**AU5′), which has a single propynyl deletion were measured and compared to those of equivalent duplexes with single dC[p]:rC or dC[p]:rA mismatches two base pairs away from the single propynyl deletion (Table 3). The d(5′C[p]**C**U[p]C[p]-C[p]U[p]U[p]3′)/r(3′GA**GGAGAA**AU5′) duplex, s-PrODN$_2$(dC[p]:rA), which has a dC[p]:rA mismatch, is 6.7 kcal/mol less stable than the fully Watson−Crick paired duplex, s-PrODN$_2$ (Table 3). The corresponding penalty is 9.1 kcal/mol for the fully propynylated PrODN:RNA duplex [compare entry 3[p](C[p]:A) in Table 2 with entry 1[p] in Table 1].

Similarly, the dC[p]:rC mismatch in the d(5′C[p]**C**U[p]C[p]C[p]-U[p]U[p]3′)/(3′rGA**GGACGAA**AU5′) duplex, s-PrODN$_2$(dC[p]:rC), is destabilizing by 7.2 kcal/mol relative to the fully Watson−Crick paired duplex (Table 3). The corresponding penalty for the fully propynylated PrODN:RNA duplex is 10.2 kcal/mol [compare entry 3[p](C[p]:C) in Table 2 with entry 1[p] in Table 1]. Differences in $\Delta\Delta G^\circ_{37}$(MM)'s within s-PrODN:RNA and the fully propynylated duplexes are remarkably similar to the 2.6 kcal/mol attributed to long-range cooperative interactions in fully propynylated duplexes with seven Watson−Crick base pairs (*39*, *40*). Apparently, long-range cooperativity between Y[p]'s enhances the thermodynamic penalties of mismatches within PrODN:RNA duplexes.

Table 2: Thermodynamic Parameters of Single Mismatch-Containing DNA:RNA, PrODN:RNA, and S-PrODN:RNA Duplexes [a]

			$1/T_m$ plots				curve-fit parameters				$\Delta\Delta G°$
entry	strands	strands	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m (°C)	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m (°C)	(MM) (kcal/mol)
dU:rG-containing duplexes											
	DNA	RNA									
1(U:G)[b]	d(5′UCUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	6.9 ± 0.1	62.1 ± 1.0	178.2 ± 3.3	38.7	6.8 ± 0.1	58.4 ± 3.8	166.4 ± 12.0	38.7	0.1
2(U:G)[b]	d(5′CUUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	5.9 ± 0.1	53.1 ± 1.6	152.2 ± 5.4	33.5	5.9 ± 0.2	55.9 ± 2.2	161.2 ± 7.2	33.3	0.9
3(U:G)[b]	d(5′CCUUCUU3′)	**r(3′**GA**GG**A**GGAA**AU5′)	6.3 ± 0.1	62.8 ± 2.4	182.4 ± 7.7	35.8	6.2 ± 0.1	69.6 ± 3.2	204.6 ± 10.6	35.6	0.5
4(U:G)[b]	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**G**AA**AU5′)	6.2 ± 0.1	62.0 ± 1.1	180.1 ± 3.5	35.2	6.2 ± 0.1	61.1 ± 5.3	177.2 ± 17.3	35.1	0.6
5(U:G)[b]	d(5′CUUCCUU3′)	**r(3′**GA**GGAGG**A**G**AU5′)	6.2 ± 0.1	54.6 ± 1.9	156.2 ± 6.2	35.1	6.2 ± 0.1	55.5 ± 1.9	158.8 ± 6.1	35.2	0.6
	PrODN	RNA									
1^P(U^P:G)[b]	d(5′UCUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	11.8 ± 0.1	59.6 ± 1.3	154.3 ± 3.8	66.8	11.8 ± 0.3	59.9 ± 3.1	155.1 ± 9.0	66.8	2.9
2^P(U^P:G)[b]	d(5′CUUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	11.2 ± 0.1	57.2 ± 1.4	148.5 ± 4.3	64.4	10.7 ± 0.2	53.0 ± 1.7	136.0 ± 4.7	64.4	4.2
3^P(U^P:G)[b]	d(5′CCUUCUU3′)	**r(3′**GA**GG**A**GGAA**AU5′)	12.3 ± 0.1	70.8 ± 1.4	188.7 ± 4.3	64.4	12.0 ± 0.2	66.8 ± 3.4	176.6 ± 10.2	64.0	2.6
4^P(U^P:G)[b]	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**G**AA**AU5′)	10.5 ± 0.3	61.9 ± 4.5	165.7 ± 13.5	58.2	10.4 ± 0.5	61.6 ± 5.9	164.8 ± 0.5	58.1	3.7
5^P(U^P:G)[b]	d(5′CUUCCUU3′)	**r(3′**GA**GGAGG**A**G**AU5′)	12.5 ± 0.2	63.0 ± 2.6	162.9 ± 7.5	69.4	13.2 ± 0.4	70.0 ± 3.3	183.4 ± 9.5	69.4	2.9
	S−PrODN	RNA									
S-1^P(U^P:G)	d(5′UCUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	12.1 ± 0.1	61.8 ± 1.7	160.2 ± 5.1	67.6	12.5 ± 0.4	67.1 ± 4.4	176.0 ± 13.0	67.4	2.4
S-2^P(U^P:G)	d(5′CUUCCUU3′)	**r(3′**GA**G**G**AGGAA**AU5′)	11.7 ± 0.2	64.7 ± 2.4	170.7 ± 7.0	64.2	12.1 ± 0.4	68.9 ± 2.8	183.3 ± 8.0	64.2	2.3
S-3^P(U^P:G)	d(5′CCUUCUU3′)	**r(3′**GA**GG**A**GGAA**AU5′)	12.7 ± 0.2	74.3 ± 2.1	198.7 ± 6.1	65.0	13.2 ± 0.5	81.0 ± 5.1	218.7 ± 15.0	64.8	1.4
S-4^P(U^P:G)	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**G**AA**AU5′)	12.0 ± 0.1	67.3 ± 1.7	178.2 ± 5.0	64.6	12.3 ± 0.4	70.1 ± 3.7	186.6 ± 10.9	64.6	2.2
S-5^P(U^P:G)	d(5′CUUCCUU3′)	**r(3′**GA**GGAGG**A**G**AU5′)	12.7 ± 0.2	68.5 ± 2.7	179.8 ± 8.0	67.9	13.4 ± 0.6	75.0 ± 5.8	198.8 ± 16.9	68.0	1.3
dC:rA-containing duplexes											
	DNA	RNA									
1(C:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AGGAA**AU5′)	6.4 ± 0.1	45.8 ± 1.1	126.9 ± 3.7	36.3	6.4 ± 0.1	51.4 ± 4.2	145.0 ± 13.5	36.1	3.2
2(C:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AAGGAA**AU5′)	(5.4 ± 0.1)	(43.8 ± 0.8)	(123.9 ± 2.5)	28.9	(5.1 ± 0.2)	(52.2 ± 5.0)	(151.8 ± 16.4)	28.6	4.2
3(C:A)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**AGAA**AU5′)	3.6 ± 0.3	48.7 ± 3.6	145.4 ± 12.4	19.6	3.6 ± 0.2	49.6 ± 3.9	148.4 ± 13.4	19.4	6.0
4(C:A)	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**A**AA**AU5′)	4.9 ± 0.1	43.1 ± 2.1	123.2 ± 7.2	25.5	4.8 ± 0.1	44.2 ± 3.9	126.8 ± 13.0	25.6	4.7
5(C:A)	d(5′CUUCCUC3′)	**r(3′**GA**GAAG**A**A**AU5′)	(6.3 ± 0.1)	(49.2 ± 1.3)	(138.5 ± 4.3)	35.4	(6.1 ± 0.1)	(58.1 ± 4.3)	(167.5 ± 13.7)	34.7	1.3
	PrODN	RNA									
1^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AGGAA**AU5′)	11.7 ± 0.1	59.0 ± 1.3	152.5 ± 3.8	66.8	11.8 ± 0.3	59.8 ± 3.1	154.8 ± 9.2	66.8	6.7
2^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AAGGAA**AU5′)	10.2 ± 0.2	58.0 ± 2.7	154.1 ± 8.2	57.7	10.0 ± 0.3	56.2 ± 5.3	149.0 ± 16.1	57.4	8.2
3^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**AGAA**AU5′)	9.3 ± 0.1	61.2 ± 2.5	167.2 ± 7.7	51.8	9.1 ± 0.2	56.2 ± 2.1	151.7 ± 6.5	52.0	9.1
4^P(C^P:A)	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**A**AA**AU5′)	(10.2 ± 0.1)	(59.0 ± 2.3)	(157.3 ± 6.9)	57.3	(9.6 ± 0.4)	(49.9 ± 4.0)	(129.9 ± 11.7)	57.3	8.2
5^P(C^P:A)	d(5′CUUCCUC3′)	**r(3′**GA**GAAG**A**A**AU5′)	12.2 ± 0.2	60.9 ± 2.3	157.0 ± 6.7	68.9	13.0 ± 0.6	69.3 ± 4.9	181.5 ± 14.0	68.0	4.0
	S−PrODN	RNA									
S-1^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AGGAA**AU5′)	(11.6 ± 0.1)	(61.7 ± 1.8)	(161.6 ± 5.3)	64.5	(10.4 ± 0.4)	(47.0 ± 3.5)	(118.0 ± 10.4)	64.7	4.2
S-2^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**A**G**AAGGAA**AU5′)	9.2 ± 0.1	60.0 ± 2.6	164.0 ± 8.1	51.2	8.9 ± 0.2	53.8 ± 2.1	114.8 ± 6.3	51.3	6.6
S-3^P(C^P:A)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**AGAA**AU5′)	8.6 ± 0.1	54.7 ± 2.0	148.7 ± 6.1	49.1	8.4 ± 0.2	47.9 ± 2.6	127.6 ± 7.9	49.4	7.2
S-4^P(C^P:A)	d(5′CCUCUUU3′)	**r(3′**GA**GGAG**A**AA**AU5′)	9.4 ± 0.1	52.3 ± 1.1	138.3 ± 3.5	54.9	9.0 ± 0.2	44.8 ± 2.6	115.6 ± 8.1	55.1	6.4
S-5^P(C^P:A)	d(5′CUUCCUC3′)	**r(3′**GA**GAAG**A**A**AU5′)	12.8 ± 0.3	67.7 ± 3.5	177.1 ± 10.3	68.7	13.7 ± 0.8	77.4 ± 7.2	205.4 ± 20.6	68.8	2.1
dC:rC containing Duplexes											
	DNA	RNA									
1(C:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	6.0 ± 0.1	44.0 ± 3.1	122.2 ± 10.3	33.6	6.0 ± 0.2	46.0 ± 3.6	128.9 ± 11.6	33.8	3.6
2(C:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	5.0 ± 0.1	42.2 ± 2.6	119.8 ± 8.8	26.3	5.2 ± 0.2	38.1 ± 3.8	105.9 ± 12.8	26.7	4.6
3(C:C)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**C**G**AA**AU5′)	(4.6 ± 0.2)	(48.5 ± 3.5)	(141.4 ± 11.7)	25.2	(5.1 ± 0.3)	(38.3 ± 2.5)	(106.9 ± 8.5)	25.9	5.0
4(C:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAG**C**AA**AU5′)	3.1 ± 0.7	42.9 ± 10.2	128.3 ± 35.1	14.2	3.3 ± 0.3	41.0 ± 5.2	121.6 ± 17.4	14.1	6.5
	PrODN	RNA									
1^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	13.7 ± 0.4	74.7 ± 3.7	196.7 ± 10.8	69.8	13.5 ± 0.8	73.9 ± 6.7	194.6 ± 19.6	69.4	4.7
2^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	8.7 ± 0.1	54.6 ± 3.4	147.9 ± 10.5	50.0	8.4 ± 0.2	47.8 ± 1.7	126.9 ± 5.0	50.0	9.7
3^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**C**G**AA**AU5′)	8.2 ± 0.1	50.9 ± 1.0	137.7 ± 3.2	47.4	8.3 ± 0.1	53.2 ± 5.2	144.9± 16.5	47.6	10.2
4^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAG**C**AA**AU5′)	(10.2 ± 0.1)	(53.4 ± 2.1)	(139.6 ± 6.4)	59.9	(10.8 ± 0.4)	(63.6 ± 4.3)	(170.1 ± 12.7)	59.6	8.2
	S−PrODN	RNA									
S-1^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	12.2 ± 0.2	66.5 ± 2.7	175.0 ± 8.1	66.2	11.6 ± 0.4	58.8 ± 3.2	152.3 ± 9.0	66.2	3.6
S-2^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**C**G**AGGAA**AU5′)	9.2 ± 0.1	49.6 ± 2.2	130.0 ± 6.9	54.9	9.1 ± 0.2	46.3 ± 3.2	119.9 ± 9.8	55.4	6.6
S-3^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GG**A**C**G**AA**AU5′)	9.8 ± 0.1	45.7 ± 1.5	115.9 ± 4.5	60.8	9.7 ± 0.1	43.4 ± 1.2	108.7 ± 3.6	61.0	6.0
S-4^P(C^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAG**C**AA**AU5′)	9.4 ± 0.1	46.7 ± 2.0	120.5 ± 6.0	57.0	9.1 ± 0.3	39.9 ± 4.5	99.3 ± 13.8	57.9	6.4
dU:rC-containing duplexes											
	DNA	RNA									
1(U:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGC**G**AA**AU5′)	4.9 ± 0.1	54.5 ± 1.6	159.8 ± 5.4	28.0	5.1 ± 0.2	50.2 ± 4.0	145.3 ± 13.4	28.4	4.7
2(U:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGC**A**A**AU5′)	7.9 ± 0.1	54.2 ± 1.4	149.2 ± 4.5	45.3	8.1 ± 0.3	61.1 ± 6.8	170.9 ± 21.0	45.2	1.7
3(U:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGA**C**A**AU5′)	8.3 ± 0.1	63.0 ± 2.3	175.9 ± 7.2	47.8	8.6 ± 0.3	63.2 ± 5.1	175.9 ± 15.9	47.5	1.3
	PrODN	RNA									
1^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGC**G**AA**AU5′)	11.5 ± 0.3	74.6 ± 4.3	203.4 ± 13.1	59.0	11.6 ± 0.4	76.0 ± 6.3	187.5 ± 18.9	59.1	6.9
2^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGC**A**A**AU5′)	14.8 ± 0.3	70.1 ± 2.7	178.5 ± 7.8	78.2	15.5 ± 0.5	77.2 ± 3.5	198.7 ± 10.0	78.0	3.6
3^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGA**C**A**AU5′)	15.6 ± 0.3	72.9 ± 2.8	184.7 ± 7.9	80.9	16.4 ± 0.3	79.8 ± 2.2	204.5 ± 6.2	80.7	2.8
	S−PrODN	RNA									
S-1^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGC**G**AA**AU5′)	10.4 ± 0.1	61.9 ± 0.6	165.8 ± 1.9	57.9	9.9 ± 0.2	52.7 ± 2.0	138.0 ± 6.1	58.3	5.4
S-2^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGC**A**A**AU5′)	13.2 ± 0.4	60.2 ± 4.0	151.6 ± 11.9	75.8	13.3 ± 0.2	61.3 ± 1.4	154.1 ± 3.9	75.6	2.6
S-3^P(U^P:C)	d(5′CCUCCUU3′)	**r(3′**GA**GGAGGA**C**A**AU5′)	14.5 ± 0.4	77.2 ± 4.0	201.9 ± 11.8	73.0	13.7 ± 0.5	68.0 ± 6.1	174.9 ± 18.1	73.7	1.3

[a] Measured in 1.0 M NaCl, 0.05 mM Na_2EDTA, 20 mM sodium cacodylate, pH 7.0. T_m listed is for 0.1 mM total strand concentration. Values in parentheses indicate the $\Delta H°$ values determined from T_M^{-1} vs $\ln(C_T/4)$ plots and from curve fitting differ by more than 15%, thus indicating non-two-state melting. Errors were calculated as described in ref *56*. [b] From ref *40*.

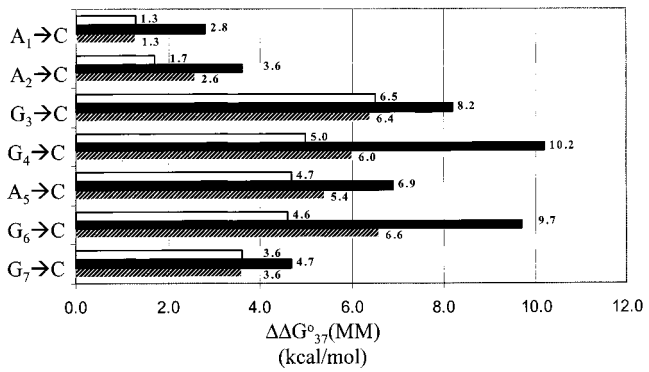

FIGURE 2: Penalties, $\Delta\Delta G^\circ_{37}$(MM)s, for rG → rC and rA → rC substitutions in the duplex (5′dC$_1$C$_2$U$_3$C$_4$C$_5$U$_6$U$_7$3′):(3′rG$_9$A$_8$**G$_7$G$_6$A$_5$G$_4$G$_3$A$_2$A$_1$**A$_{-1}$U$_{-2}$5′), creating dC:rC and dU:rC mismatches in DNA:RNA (white), PrODN:RNA (black), and S-PrODN:RNA duplexes (striped). On average, propynylation increases $\Delta\Delta G^\circ_{37}$(MM) for dC:rC and dU:rC mismatches by 3.3 and 1.9 kcal/mol, respectively, when the backbone is phosphodiester. Full stereorandom phosphorothioate substitution decreases the average propynylation effect, $\Delta\Delta G^\circ_{37}$(MM), for dC:rC and dU:rC mismatches to 0.7 and 0.5 kcal/mol, respectively.

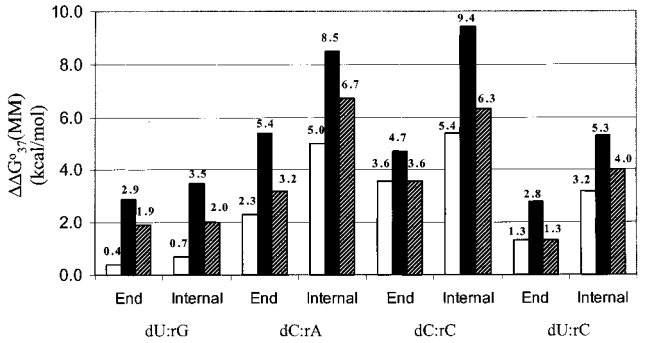

FIGURE 3: Average $\Delta\Delta G^\circ_{37}$(MM)s for dU:rG, dC:rA, dC:rC, and dU:rC at terminal and internal positions within DNA:RNA (white), PrODN:RNA (black), and S-PrODN:RNA (striped) duplexes.

DISCUSSION

Specificity of nucleic acid hybridization is important for designing antisense and probe oligonucleotides. Specificity is typically reduced as oligomer length is increased and free energies per base pair are made more favorable (*57*, *58*). It is interesting, therefore, that PrODNs have more favorable free energies for base pairing (*37*−*40*), but are specific to RNA targets (*41*−*44*). The results reported here suggest that the specificity at least partially arises from enhanced penalties for mismatches in hybrid propynylated duplexes.

Effects of Full Propynylation on Specificity. The order of mismatch penalties in DNA:RNA duplexes is dC:rC-internal ∼ dC:rA-internal ≫ dC:rC-end ∼ dU:rC-internal > dC:rA-end > dU:rC-end > dU:rG-internal ∼ dU:rG-end (Figure 3). The order of mismatch penalties in PrODN:RNA duplexes is dC:rC-internal > dC:rA-internal ≫ dC:rA-end ∼ dU:rC-internal ∼ dC:rC-end > dU^p:rG-internal ∼ dU^p:rG-end ∼ dU^p:rC-end (Figure 3). Thus, the order of mismatch penalties is similar for DNA:RNA and PrODN:RNA duplexes, even though the magnitudes of penalties are larger with PrODNs.

Full propynylation enhances single mismatch penalties by 1.1−4.0 kcal/mol (Figure 3). The average $\Delta\Delta G^\circ_{37}$(MM-end) and $\Delta\Delta G^\circ_{37}$(MM-internal) changes by 2.1 and 3.1 kcal/mol, respectively, upon full propynylation of DNA:RNA duplexes (Figure 3), and the effect enhances the destabilizing penalties of all internal and terminal single mismatches studied. The order of enhanced destabilization is dC^p:rC-internal ∼ dC^p:rA-internal > dC^p:rA-end ∼ dU^p:rG-internal > dU^p:rG-end ∼ dU^p:rC-internal > dU^p:rC-end ∼ dC^p:rC-end. Some mismatches with the least destabilizing $\Delta\Delta G^\circ_{37}$(MM)'s in DNA:RNA duplexes, such as dU^p:rG, are highly destabilized upon full propynylation. This provides a way to enhance specificity in nucleic acid hybridization. Other mismatches that are more destabilizing to DNA:RNA duplexes, such as dU:rC-internal and dU:rC-end, are destabilized the least upon full propynylation. The net result is that PrODN:RNA duplexes provide large penalties for all mismatches studied, whereas DNA:RNA duplexes do not.

The magnitude by which mismatch penalties are enhanced is position-dependent. On average, terminal mismatch penalties are enhanced less than internal mismatch penalties in PrODN:RNA duplexes (Figure 3). Upon full propynylation, penalties for internal dU:rG, dU:rC, and dC:rA mismatches are enhanced by only 0.3−0.6 kcal/mol relative to their equivalent terminal mismatches. In contrast, the penalty for an internal dC:rC mismatch is enhanced an average of 2.9 kcal/mol more than that of a terminal dC:rC mismatch upon full propynylation. The uniqueness of dC:rC mismatches could be related to the fact that C:C mismatches are less likely to hydrogen bond than U:G, U:C, and C:A mismatches, which can form hydrogen bonds at neutral pH (*59*−*61*).

Long-Range Cooperative Interactions of Propynyls Enhance Mismatch Penalties. Full propynylation of DNA:RNA duplexes increases ΔG°_{37}(MM)'s by an average of 2.9 kcal/mol (Table 2). This increment is remarkably similar to the 2.6 kcal/mol of stability provided by a cooperativity bonus in a model for prediction of the stabilities of PrODN:RNA duplexes with seven Watson−Crick base pairs (*39*). These results suggest that the enhanced mismatch penalty for fully propynylated duplexes results from loss of long-range cooperative interactions between Y^p's when mismatches are present.

Deleting a single propynyl group from a Y^p in a Watson−Crick pair can eliminate the cooperative interaction of propynyls in an entire 7-mer duplex (*39*). Deleting a single propynyl group in a Watson−Crick pair two base pairs away from a dU^p:rG pair reduces $\Delta\Delta G^\circ_{37}$(dU^p:rG) by 2.6 kcal/mol (*40*). This suggests the enhanced $\Delta\Delta G^\circ_{37}$(MM) results from long-range cooperative interactions between Y^p's (*39*, *40*). Tables 2 and 3 reveal a similar effect for dC^p:rC and dC^p:rA mismatches. In these cases, deleting a single propynyl group two base pairs from the position of the mismatch reduces $\Delta\Delta G^\circ_{37}$(MM) by 2.4 and 3.0 kcal/mol, respectively (Tables 2 and 3). Evidently, the enhanced $\Delta\Delta G^\circ_{37}$(MM) increments in PrODN:RNA duplexes depend on the cooperativity between propynylated pyrimidines.

Effects of Full Phosphorothioate Substitution on Specificity. The order of mismatch penalties within S-PrODN:RNA duplexes is dC:rA-internal ∼ dC:rC-internal ≫ dU:rC-internal ∼ dC:rC-end > dC:rA-end > dU^p:rG-internal ∼ dU^p:rG-end > dU^p:rC-end, which is similar to those observed with DNA:RNA and PrODN:RNA helices (Figure 3). With the exception of $\Delta\Delta G^\circ_{37}$(dU:rC-end) and $\Delta\Delta G^\circ_{37}$(dC:rC-end), $\Delta\Delta G^\circ_{37}$(MM)'s for S-PrODN:RNA duplexes are more destabilizing than those for DNA:RNA duplexes.

Table 3: Thermodynamic Parameters of s-PrODN$_2$:RNA Duplexes Possessing dC$_4$:rC$_4$ and dC$_4$:rA$_4$ Mismatches[a]

			$1/T_m$ plots				curve-fit parameters				
entry	PrODN	RNA	$-\Delta G^\circ_{37}$ (kcal/mol)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	T_m (°C)	$-\Delta G^\circ_{37}$ (kcal/mol)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	T_m (°C)	$\Delta\Delta G^\circ$(MM) (kcal/mol)
s-PrODN$_2$[b]	d(5′C^p**C**U^{p}C^{p}C^{p}U^{p}U^{p}3′)	**r**(3′GA**GGAGGAA**AU5′)	14.8 ± 0.2	70.8 ± 2.0	180.6 ± 4.9	78.2	14.9 ± 0.4	71.4 ± 4.1	182.3 ± 10.3	78.2	
s-PrODN$_2$ (dC^p:rA)	d(5′C^p**C**U^{p}C^{p}C^{p}U^{p}U^{p}3′)	**r**(3′GA**GGAAGAA**AU5′)	8.1 ± 0.1	52.2 ± 1.3	142.3 ± 4.1	46.3	8.0 ± 0.2	48.2 ± 3.9	129.6 ± 12.2	46.5	6.7
s-PrODN$_2$ (dC^p:rC)	d(5′C^p**C**U^{p}C^{p}C^{p}U^{p}U^{p}3′)	**r**(3′GA**GGACGAA**AU5′)	7.6 ± 0.1	62.6 ± 1.8	177.2 ± 5.9	42.3	7.5 ± 0.1	55.2 ± 1.8	153.7 ± 5.6	42.4	7.2

[a] Measured in 1.0 M NaCl, 0.05 mM Na$_2$EDTA, 20 mM sodium cacodylate, pH 7.0. Errors are based on the standard deviations of the thermodynamic parameters and were calculated as described in ref *56*. T_m listed is for 0.1 mM total strand concentration. [b] From ref *39*.

Full stereo-random phosphorothioate substitution of PrODN:RNA duplexes reduces all mismatch penalties, with the average $\Delta\Delta G^\circ_{37}$(MM-end) and $\Delta\Delta G^\circ_{37}$(MM-internal) penalties reduced by 1.4 and 1.9 kcal/mol, respectively. The order of reduction of ΔG°_{37}(MM)'s upon stereo-random phosphorothioate substitution of PrODN:RNA duplexes is dC^p:rC-internal ≫ dC^p:rA-end ∼ dC^p:rA-internal > dU^p:rG-internal ∼ dU^p:rC-end ∼ dU^p:rC-internal > dC^p:rC-end ∼ dU^p:rG-end (Figure 3). Interestingly, the order of reduction is similar to the order of enhancement in mismatch penalties due to propynylation.

The magnitude by which mismatch penalties are reduced by phosphorothioate substitution depends on whether the mismatch is terminal or internal (Figure 3). Most dramatically, for a dC^p:rC mismatch, $\Delta\Delta G^\circ_{37}$(MM-end) is reduced by only 1.1 kcal/mol, while $\Delta\Delta G^\circ_{37}$(MM-internal) is reduced by 3.1 kcal/mol (Figure 3). The magnitudes of mismatch penalty reduction for all other mismatches are much less position-dependent. Interesting, for dC^p:rC and dU^p:rG mismatches, $\Delta\Delta G^\circ_{37}$(MM-internal) is reduced more than $\Delta\Delta G^\circ_{37}$(MM-end) upon phosphorothioate substitution of PrODN:RNA duplexes. In contrast, for dU^p:rC and dC^p:rA mismatches, $\Delta\Delta G^\circ_{37}$(MM-internal) is reduced less than $\Delta\Delta G^\circ_{37}$(MM-end).

Effects of Full Phosphorothioate Substitution on PrODN:RNA Stability. The T_m's of Watson−Crick PrODN:RNA duplexes differ from those of S-PrODN:RNA duplexes by an average of only 2.3 °C, and on average, the PrODN:RNA duplexes are more stable by 1.3 kcal/mol at 37 °C (Table 1). These trends have been observed previously for unpropynylated DNA:RNA and stereo-regular S-DNA:RNA duplexes (*62, 63*). In general, the stability change per phosphorothioate substitution is modest.

The two 5′ and two 3′ unpaired ribonucleotides in the S-PrODN:RNA-1^p duplex increase duplex stability by 3.0 kcal/mol over the S-PrODN:RNA-7^p duplex. This thermodynamic contribution is similar to that found with the equivalent PrODN:RNA duplexes (3.1 kcal/mol), but more favorable than with the DNA:RNA duplexes (2.0 kcal/mol) (*39*). Evidently, the helical geometry affording enhanced cross-strand stacking interactions in PrODN:RNA duplexes is retained in S-PrODN:RNA duplexes. The circular dichroism spectra of S-PrODN-1^p (unpublished results) and its phosphodiester analogue (*39*) are very similar, supporting the idea of similar helical geometries. These findings are consistent with those reported for DNA:RNA and stereo-random S-DNA:RNA duplexes (*62*).

Comparison of PrODN:RNA Duplex Stabilities with Predictions of a Cooperative Model. A cooperative model has been presented that accounts for the stability enhancements of PrODN:RNA duplexes (*39*). Table 1 shows the free energy changes predicted for the PrODN:RNA duplexes studied. The rmsd between the measured and predicted ΔG°_{37} is 0.74 kcal/mol, which is only 4.7% that of the average measured ΔG°_{37} for these duplexes. Thus, the cooperativity model (*39*) is very good at predicting the stabilities of these PrODN:RNA duplexes.

Comparison of DNA:RNA Duplex Stabilities with Predictions. Sugimoto et al. reported thermodynamics for many DNA:RNA duplexes, and derived parameters for the prediction of DNA:RNA duplex stability on the basis of an individual nearest-neighbor model (INN) (*64*). Gray used the same data to derive parameters for the prediction of DNA:RNA duplex stability on the basis of an independent short sequence model (ISS) (*65*). After accounting for unpaired terminal nucleotides, the results reported in Table 1 for DNA:RNA duplexes can be compared with predictions if corrections are applied for the expected difference between dU and dT. Studies have shown that the C5-methyl group renders T:A pairs 0.3 kcal/mol more stable, on average, than U:A pairs (*30, 66*). As shown in Table 1, after corrections, the predictions correspond very well with experimental values ($rmsd_{INN}$ = 0.45 kcal/mol and $rmsd_{ISS}$ = 0.64 kcal/mol). Evidently, combining the INN or ISS models with the 0.3 kcal/mol penalty for changing dT to dU in DNA results in an accurate prediction of stabilities of Watson−Crick paired DNA:RNA duplexes. The agreement between measured and predicted stabilities of DNA:RNA duplexes and stability enhancements of PrODN:RNA duplexes suggests that the stabilities of PrODN:RNA duplexes can be predicted by using INN (*64*) or ISS (*65*) models and the dT to dU correction (*30, 66*) to calculate the stability of a DNA:RNA duplex, and then using the long-range cooperativity model to predict the effects of various degrees of propynylation (*39*).

Comparison of $\Delta\Delta G^\circ_{37}$(MM)'s for DNA:RNA Duplexes with Nearest-Neighbor Model Predictions. The $\Delta\Delta G^\circ_{37}$(MM) values in Table 2 can be compared with expectations from average trinucleotide parameters (*30*). For example, Sugimoto et al. determined free energies for single internal dC:rA mismatches within all four permutations of G:C/C:G trinucleotide contexts (*30*). The values range from −0.7 to +0.9 kcal/mol and average −0.2 kcal/mol. After correcting for the loss of a dC:rG base pair on the basis of free energies of G:C/C:G nearest-neighbor interactions (*64*), the model predicts an average 4.8 kcal/mol mismatch penalty for dC:rA in a DNA:RNA duplex. The average $\Delta\Delta G^\circ_{37}$(MM-internal) for dC:rA in Table 2 is 5.0 kcal/mol (Figure 3), which is within experimental error of the predicted value.

Similarly, the average $\Delta\Delta G^\circ_{37}$(MM) for a single internal dU:rG pair in a DNA:RNA duplex is 0.2 kcal/mol as

calculated for permutations of G:C/C:G trinucleotide contexts (*30*, *64*). The average $\Delta\Delta G^\circ_{37}$(MM-internal) in Table 2 for dU:rG is 0.7 kcal/mol (Figure 3), again within experimental error of the predicted value. Evidently, $\Delta\Delta G^\circ_{37}$(MM)'s for dC:rA and dU:rG can be predicted roughly from a nearest-neighbor model even though the $\Delta\Delta G^\circ_{37}$(MM)'s determined here were generated within different nearest-neighbor motifs.

Similar comparisons cannot be made for dU:rC and dC:rC mismatches because they were too destabilizing to be measured in a previous study (*30*). The DNA:RNA duplexes with dU:rC and dC:rC mismatches in Table 2 are more stable than those studied previously, and provide average values of 3.2 and 5.4 kcal/mol, respectively, for the $\Delta\Delta G^\circ_{37}$(MM)'s of dU:rC and dC:rC internal mismatches (Figure 3).

Comparing Mismatch Specificity of Linear PrODNs to Circular Oligonucleotides. Circular oligonucleotides that can recognize bases by both Watson−Crick base pair and base triple formation provide some of the tightest binding and the highest specificity previously observed for nucleic acid hybridizations (*31*−*33*, *67*, *68*). For example, at pH 7.0 and 25 °C, the DNA 12-mer (5′dAAGAAAGAAAAG3′) binds to a 34-mer circular DNA to give $\Delta G^\circ_{25} = -18.1$ kcal/mol with a range in $\Delta\Delta G^\circ_{37}$(MM-internal) of 7.1−7.5 kcal/mol (*33*). At pH 5.5, binding and specificity are enhanced by formation of additional base triples (C^+·G-C) to give $\Delta G^\circ_{25} = -25.5$ kcal/mol with a range in $\Delta\Delta G^\circ_{37}$(MM-internal) of 8.2−10.4 kcal/mol (*33*). The results in Tables 1 and 2 and Figure 3 show that propynylated oligopyrimidine 7-mers provide similar binding and specificity with RNA 11-mers. The results suggest that a variety of approaches can be used to provide the specificity and tight binding that will optimize the many emerging applications of nucleic acid hybridizations.

ACKNOWLEDGMENT

We thank Drs. M. D. Matteucci and B. C. Froehler for sparking our interest in these systems, and Professor Stephen M. Testa in the Department of Chemistry at the University of Kentucky for stimulating discussions.

SUPPORTING INFORMATION AVAILABLE

Sample UV melting curves and $1/T_M$ vs $\log(C_T/4)$ plots. Three tables of thermodynamic values obtained by averaging fits of individual curves (14 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Zamecnik, P. C., and Stephenson, M. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 280−284.
2. Milligan, J. F., Matteucci, M. D., and Martin, J. C. (1993) *J. Med. Chem. 36*, 1923−1937.
3. Agrawal, S., and Iyer, R. P. (1997) *Pharmacol. Ther. 76*, 151−160.
4. Pearson, N. D., and Prescott, C. D. (1997) *Chem. Biol. 4*, 409−414.
5. Branch, A. D. (1998) *Trends Biochem. Sci. 23*, 45−50.
6. Crooke, S. T. (2000) *Methods Enzymol. 313*, 3−45.
7. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) *Science 270*, 467−470.
8. Healey, B. G., Matson, R. S., and Walt, D. R. (1997) *Anal. Biochem. 251*, 270−279.
9. Hacia, J. G., Woski, S. A., Fidanza, J., Edgemon, K., Hunt, N., McGall, G., Fodor, A. P. A., and Collins, F. S. (1998) *Nucleic Acids Res. 26*, 4975−4982.
10. Maldonado-Rodriguez, R., Espinosa-Lara, M., Loyola-Abitia, P., Beattie, W. G., and Beattie, K. L. (1999) *Mol. Biotechnol. 11*, 13−25.
11. Gerry, N. P., Witowski, N. E., Day, J., Hammer, R. P., Barany, G., and Barany, F. (1999) *J. Mol. Biol. 292*, 251−262.
12. Chen, D., Yan, Z., Cole, D. L., and Srivatsa, G. S. (1999) *Nucleic Acids Res. 27*, 389−395.
13. Walt, D. R. (2000) *Science 287*, 451−452.
14. Gralla, J., and Crothers, D. M. (1973) *J. Mol. Biol. 78*, 310−319.
15. Peritz, A. E., Kierzek, R., Sugimoto, N., and Turner, D. H. (1991) *Biochemistry 30*, 6428−6436.
16. SantaLucia, J., Jr., Kierzek, R., and Turner, D. H. (1991) *Biochemistry 30*, 8242−8251.
17. Morse, S. E., and Draper, D. E. (1995) *Nucleic Acids Res. 23*, 302−306.
18. Zhu, J., and Wartell, R. M. (1997) *Biochemistry 36*, 15326−15335.
19. Bevilacqua, J. M., and Bevilacqua P. C. (1998) *Biochemistry 37*, 15877−15884.
20. Meroueth, M., and Chow, C. S. (1999) *Nucleic Acids Res. 27*, 1118−1125.
21. Kierzek, R., Burkard, M. E., and Turner, D. H. (1999) *Biochemistry 38*, 14214−14223.
22. Leonard, G. A., Booth, E. D., and Brown, T. (1990) *Nucleic Acids Res. 18*, 5617−5623.
23. Otikiti, E. O., and Sheardy, R. D. (1997) *Biochemistry 36*, 11419−11427.
24. Zhong, M., Marky, L. A., Kallenbach, N. R., and Kupke, D. W. (1997) *Biochemistry 36*, 2485−2491.
25. Allawi, H. T., and SantaLucia, J., Jr. (1997) *Biochemistry 36*, 10581−10594.
26. Allawi, H. T., and SantaLucia, J., Jr. (1998) *Biochemistry 37*, 9435−9444.
27. Allawi, H. T., and SantaLucia, J., Jr. (1998) *Nucleic Acids Res. 26*, 2694−2701.
28. Allawi, H. T., and SantaLucia, J., Jr. (1998) *Biochemistry 37*, 2170−2179.
29. Peyret, N., Seneviratne, P. A., Allawi, H. T., and SantaLucia, J., Jr. (1999) *Biochemistry 38*, 3468−3477.
30. Sugimoto, N., Nakano, M., and Nakano, S. (2000) *Biochemistry 39*, 11270−11281.
31. Kool, E. T. (1991) *J. Am. Chem. Soc. 113*, 6265−6266.
32. Wang, S., and Kool, E. T. (1994) *Nucleic Acids Res. 22*, 2326−2333.
33. Wang, S., Friedman, A. E., and Kool, E. T. (1995) *Biochemistry 34*, 9774−9784.
34. Testa, S. M., Disney, M. D., Turner, D. H., and Kierzek, R. (1999) *Biochemistry 38*, 16655−16662.
35. De Clercq, E., Descamps, J., Balzarini, J., Giziewicz, J., Barr, P. J., and Robins, M. J. (1983) *J. Med. Chem. 26*, 661−666.
36. Hobbs, F. W. (1989) *J. Org. Chem. 54*, 3420−3422.
37. Froehler, B. C., Wadwani, S., Terhorst, T. J., and Gerrard, S. R. (1992) *Tetrahedron Lett. 33*, 5307−5310.
38. Freier, S. M., and Altmann, K. H. (1997) *Nucleic Acids Res. 25*, 4429−4443.
39. Barnes, T. W., and Turner, D. H. (2001) *J. Am. Chem. Soc. 123*, 4107−4118.
40. Barnes, T. W., and Turner, D. H. (2001) *J. Am. Chem. Soc. 123*, 9186−9187.
41. Wagner, R. W., Matteucci, M. D., Lewis, J. G., Guitierrez, A. J., Moulds, C., and Froehler, B. C. (1993) *Science 260*, 1510−1513.
42. Moulds, C., Lewis, J. G., Froehler, B. C., Grant, D., Huang, T., Milligan, J. F., Matteucci, M. D., and Wagner, R. W. (1995) *Biochemistry 34*, 5044−5053.
43. Flanagan, W. M., Kothavale, A., and Wagner, R. W. (1996) *Nucleic Acids Res. 24*, 2936−2941.
44. Wagner, R. W., Matteucci, M. D., Grant, D., Huang, T., and Froehler, B. C. (1996) *Nat. Biotechnol. 14*, 840−844.

45. Usman, N., Ogilvie, K. K., Jiang, M.-V., and Cedergren, R. (1987) *J. Am. Chem. Soc. 109*, 7845−7854.
46. Matteucci, M. D., and Caruthers, M. H. (1981) *BioTechnology 24*, 92−98.
47. Wincott, F., DiRenzo, A., Shaffer, C., Grimm, S., Tracz, D., Workman, C., Sweedler, D., Gonzalez, C., Scaringe, S., and Usman, N. (1995) *Nucleic Acids Res. 23*, 2677−2684.
48. Iyer, R. P., Egan, W., Regan, J. B., and Beaucage, S. L. (1990) *J. Am. Chem. Soc. 112*, 1253−1254.
49. Stawinski, J., Strömberg, R., Thelin, M., and Westman, E. (1988) *Nucleosides Nucleotides 7*, 779−782.
50. Borer, P. N. (1975) in *Handbook of Biochemistry and Molecular Biology: Nucleic Acids* (Fasman, G. D., Ed.) 3rd ed., Vol. I, p 589, CRC Press, Cleveland, OH.
51. Richards, E. G. (1975) in *Handbook of Biochemistry and Molecular Biology: Nucleic Acids* (Fasman, G. D., Ed.) 3rd ed., Vol. I, p 597, CRC Press, Cleveland, OH.
52. Puglisi, J. D., and Tinoco, I., Jr. (1989) *Methods Enzymol. 180*, 304−325.
53. Longfellow, C. E., Kierzek, R., and Turner, D. H. (1990) *Biochemistry 29*, 278−285.
54. McDowell, J. A., and Turner, D. H (1996) *Biochemistry 35*, 14077−14089.
55. Borer, P. N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O. C. (1974) *J. Mol. Biol. 241*, 246−262.
56. Xia, T., SantaLucia, J., Jr., Burkard, M. E., Kierzek, R., Schroeder, S. J., Jiao, X., Cox, C., and Turner, D. H. (1998) *Biochemistry 37*, 14719−14735.
57. Herschlag, D. (1991*) Proc. Natl. Acad. Sci. U.S.A. 88*, 6921−6925.
58. Roberts, R. W., and Crothers, D. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9397−9401.
59. Hare, D., Shapiro, L., and Patel, D. J. (1986) *Biochemistry 25*, 7445−7456.
60. Tanaka, Y., Kojima, C., Yamazaki, T., Kodama, T. S., Yasuno, K., Miyashita, S., Ono, A., Ono, A., Kainosho, M., and Kyogoku, Y. (2000) *Biochemistry 39*, 7074−7080.
61. Pan, B., Mitra, S. N., and Sundaralingam, M. (1998) *J. Mol. Biol. 283*, 977−984.
62. Clark, C. L., Cecil, P. K., Singh, D., and Gray, D. M. (1997) *Nucleic Acids Res. 25*, 4098−4105.
63. Hashem, G. M., Pham, L., Vaughan, M. R., and Gray, D. M. (1998) *Biochemistry 37*, 61−72.
64. Sugimoto, N., Nakano, S., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M., and Sasaki, M. (1995) *Biochemistry 34*, 11211−11216.
65. Gray, D. M. (1997) *Biopolymers 42*, 795−810.
66. Wang, S., and Kool, E. T. (1995) *Biochemistry 34*, 4125−4132.
67. Prakash, G., and Kool, E. T. (1991) *J. Chem. Soc., Chem. Commun.*, 1161−1162.
68. Wang, S., and Kool, E. T. (1995) *Nucleic Acids Res. 23*, 1157−1164.

BI011033+